

Regulation of expression by promoters versus internal ribosome entry site in the 5'-untranslated sequence of the human cyclin-dependent kinase inhibitor p27^{kip1}

Zhaoqian Liu, Zizheng Dong, Baoguang Han, Youyun Yang, Yang Liu and Jian-Ting Zhang*

Department of Pharmacology and Toxicology, Indiana University Cancer Center, Walther Oncology Center/Walther Cancer Institute, Indiana University School of Medicine, 1044 W. Walnut Street, Indianapolis, IN 46202, USA

Received as resubmission May 15, 2005; Accepted June 12, 2005

ABSTRACT

p27^{kip1} regulates cell proliferation by binding to and inhibiting the activity of cyclin-dependent kinases and its expression oscillates with cell cycle. Recently, it has been suggested from studies using the traditional dicistronic DNA assay that the expression of p27^{kip1} is regulated by internal ribosome entry site (IRES)-mediated translation initiation, and several RNA-binding protein factors were thought to play some role in this regulation. Considering the inevitable drawbacks of the dicistronic DNA assay, which could mislead a promoter activity or alternative splicing to IRES as previously demonstrated, we decided to reanalyze the 5'-untranslated region (5'-UTR) sequence of p27^{kip1} and test whether it contains an IRES element or a promoter using more stringent methods, such as dicistronic RNA and promoterless dicistronic and monocistronic DNA assays. We found that the 5'-UTR sequence of human p27^{kip1} does not have any significant IRES activity. The previously observed IRES activities are likely generated from the promoter activities present in the 5'-UTR sequences of p27^{kip1}. The findings in this study indicate that transcriptional regulation likely plays an important role in p27^{kip1} expression, and the mechanism of regulation of p27 expression by RNA-binding factors needs to be re-examined. The findings in this

study also further enforce the importance that more stringent studies, such as promoterless dicistronic and monocistronic DNA and dicistronic RNA tests, are required to safeguard any future claims of cellular IRES.

INTRODUCTION

p27^{kip1} (referred as p27) is an inhibitory protein of cyclin-dependent kinases (Cdks) and it plays a key role in regulating Cdk activity during cell cycle progression and in growth arrest (1,2). The expression level of p27 is a critical determinant for the decision of cells in G1 to either withdraw from or commit to the cell cycle and enter S phase. The expression of p27 oscillates with cell cycle and is higher in G1 and lower in other phases (3).

The regulation of p27 expression is precise and has been suggested to occur at many levels, including translation initiation (4–6). In 1996, Agrawal *et al.* (4) and Hengst and Reed (5) found that the expression of p27 may be regulated at the translational level. Millard *et al.* (7) found that the translation of p27 mRNA in both basal (proliferation) and induced (nonproliferation) states required a U-rich sequence in the 5'-untranslated region (5'-UTR). Using a dicistronic DNA vector, Miskimins *et al.* (8) found that an internal ribosome entry site (IRES) element present in the 217 bases of the 5'-UTR of mRNAs may be involved in the translational regulation of murine p27. Using similar strategies, the IRES in the 5'-UTR of human p27, however, was thought to be longer and

*To whom correspondence should be addressed. Tel: +1 317 278 4503; Fax: +1 317 274 8046; Email: jianzhan@iupui.edu

Present address:

Zhaoqian Liu, Institute of Clinical Pharmacology, Central South University Xiangya School of Medicine, Changsha, People's Republic of China 410078

The authors wish to be known that, in their opinion, the first two authors should be regarded as joint First Authors

© The Author 2005. Published by Oxford University Press. All rights reserved.

The online version of this article has been published under an open access model. Users are entitled to use, reproduce, disseminate, or display the open access version of this article for non-commercial purposes provided that: the original authorship is properly and fully attributed; the Journal and Oxford University Press are attributed as the original place of publication with the correct citation details given; if an article is subsequently reproduced or disseminated not in its entirety but only in part or as a derivative work this must be clearly indicated. For commercial re-use, please contact journals.permissions@oupjournals.org

it may consist of 356 bases (9). Recently, it was shown that an upstream open reading frame (ORF) in the 5'-UTR of human p27 is important for the IRES activity (10).

Initiation of translation of most eukaryotic mRNAs normally depends on the 5' m⁷GpppN cap structure of mRNAs, which recruits 43S ribosome preinitiation complex via interaction with the cap binding protein eIF4E (11). The translation machinery then migrates downstream until it meets the first AUG codon in the optimal context for initiation of translation (12,13). This scanning model implies that any mRNAs with long 5'-UTRs and complex secondary structures may not be translated efficiently. An alternative mechanism, IRES-mediated translation initiation, has thus been proposed. With this mechanism, ribosomes can be directly recruited to an internal site in a 5'-UTR sequence proximal to the AUG start codon for initiation without the use of the 5'-cap structure. Up to date, many eukaryotic cellular mRNAs have been suggested to contain such IRES activities (see <http://www.rangueil.inserm.fr/IRESdatabase>). While it is possible that some cellular mRNAs may use the IRES mechanism to initiate translation (14), their existence has been challenged due to the inevitable drawbacks of the conventional dicistronic DNA assay used in the majority of these previous studies (15–17). Despite such concerns, many new studies claiming cellular IRES are being published without any rigorous analysis to rule out the potential interference of promoters and alternative splicing events.

In the present study, we re-examined the 5'-UTR sequence of human p27 mRNA and further tested the putative IRES activities using promoterless dicistronic and monocistronic vectors and direct assays of dicistronic mRNAs. Using these stringent assays, we found that the 5'-UTR sequence of human p27 does not have IRES activities but contains promoters which may be involved in regulating p27 expression. These promoter activities are likely responsible for the previously reported putative IRES activities of p27.

MATERIALS AND METHODS

Materials

Restriction endonucleases and *Pfu* polymerase were purchased from New England Biolabs and Stratagene, respectively. Sp6 and T7 RNA polymerases, RNasin, RNase-free DNase I, Luciferase Reporter Assay Systems, pGEM-T easy and pSP64 poly(A) vectors were from Promega. RNeasy kit and Oligotex mRNA Mini kit were from Qiagen. MAXIscript *in vitro* transcription kit and RPA III Ribonuclease Protection Assay Kit were from Ambion. m⁷GpppG cap analog and [α -³²P]dCTP were from Amersham/Pharmacia. The Sephadex G-25 Quick Spin Columns (TE) for radio-labeled DNA and RNA purification were from Roche Diagnostics. PCR Cloning kit, cell culture media and sera, Lipofectamine Plus and Lipofectin transfection reagents were all from Invitrogen. All other reagents were of molecular biology grade from Fisher or Sigma.

Construct engineering

The full-length sequence of 5'-UTR of human p27 is 575 bases (9). The full-length and the two deletion mutants of 5'-UTR

sequences of human p27 were amplified from human genomic DNA using a common reverse primer 5'-GGCCATGGC-TTTCTCCCGGGTCTGCACGA-3' and the following individual forward primers: 5'-GGACTAGTCCACCTTAAG-GCCGCGCTCGC-3' for full-length (–575 to –1) 5'-UTR; 5'-GGACTAGTAGCCTCCCTTCCACCGCCATA-3' for deletion construct (–461 to –1); and 5'-GGACTAGTGGCCGTG-GCTCGTCGGGGTCT-3' for deletion construct (–150 to –1). These PCR products were then cloned into pGEM-T Easy vector.

To engineer dicistronic constructs containing different 5'-UTRs of human p27, the dicistronic vector pRF that contains *Renilla* and firefly luciferase genes (18) was used. The full-length and the two deletion constructs of human p27 5'-UTR in pGEM-T Easy were released by digestion with SpeI and NcoI and subsequently cloned into pRF vector to obtain pR-p27-F(–575), pR-p27-F(–461) and pR-p27-F(–150). The promoterless dicistronic constructs containing these 5'-UTRs were engineered by removing the vector SV40 promoter together with the chimeric intron as described previously (19). The hairpin structure in the dicistronic constructs were engineered by cloning the 5'-UTR sequences into the dicistronic constructs containing a hairpin prepared in a previous study (20). To generate constructs that can be used for *in vitro* production of transcripts with poly(A) tail, the 5'-UTR sequences in the dicistronic constructs were released by digestion with SpeI and NcoI and subsequently cloned into the pSP-R-HRV-F_{A30} plasmid (19) by replacing the HRV IRES sequence digested with SpeI and NcoI.

In vitro transcription

In vitro transcription was performed as described previously (21). Briefly, pRFA₃₀-based plasmids were linearized by EcoRI and the capped transcripts were synthesized *in vitro* in the presence of 1 mM m⁷GpppG. The DNA template was then removed by digestion using RNase-free DNase I, and the RNA transcripts were purified using a Qiagen RNeasy Mini kit.

Purification of recombinant human polypyrimidine tract-binding protein (PTB) and *in vitro* translation

His-tagged PTB was expressed in *Escherichia coli* BL21(DE3) cells using the pET28aPTB construct (22) and purified using Ni-NTA Sepharose (Qiagen) according to the supplier's protocol. The purified His-PTB was extensively dialyzed against H100 buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride and 10 μ g/ml leupeptin) prior to use in the *in vitro* translation system.

In vitro translation was performed as described previously (19,20,23). Briefly, 100 ng of capped dicistronic RNA transcripts were used to program cell-free translation in rabbit reticulocyte lysate (RRL) in a final volume of 10 μ l containing 6.5 μ l RRL. The translation mixture contained either 1 μ l of H100 buffer (control) or 1 μ l of purified His-PTB at 25 μ g/ml.

Cell culture and transfections

Human large cell lung cancer cell line H1299 and human embryonic kidney 293T cells were maintained in RPMI 1640 and DMEM media, respectively, supplemented with 10%

fetal bovine serum in humidified incubators at 37°C with 5% CO₂.

Transient transfection with DNA constructs were performed with Lipofectamine plus reagents as described previously (19,20,23). Briefly, $\sim 6 \times 10^4$ cells/well in a 24-well plate were co-transfected with 0.4 µg of test DNA and 50 ng of β-galactosidase control DNA. Cells were then harvested 24 h post-transfection followed by analysis of luciferase and galactosidase activities.

Transient transfection with RNA transcripts were also performed using cationic liposomes as described previously (19,20,23). Briefly, $\sim 3 \times 10^5$ cells/well were seeded onto 6-well plates on the day before transfection. Cells were washed once with Opti-MEM I reduced serum medium (Invitrogen) and left in the incubator with some medium during preparation of the liposome-polynucleotide complexes. One milliliter of Opti-MEM I medium in a 12 × 75 mm polystyrene snap-cap tube was mixed with 12.5 µg of Lipofectin reagent and 5 µg of RNA transcripts. The liposome–RNA–medium mixture was immediately added to cells. Eight hours following transfection, cells were harvested and processed for luciferase assay.

Luciferase reporter assay

Cells co-transfected with dicistronic or monocistronic reporter plasmids and β-galactosidase control plasmid were lysed in the Passive Lysis Buffer (Promega). Both the firefly (Fluc) and *Renilla* luciferase (Rluc) activities were determined using the Dual-Luciferase Reporter Assay kit as described previously (19). The firefly luciferase activity from monocistronic constructs was determined using the Mono-Luciferase Reporter Assay kit. The activity of β-galactosidase was measured as described previously (24) and was used to normalize the differences of transfection efficiency.

RNA extraction and RNase protection assay

Approximately 2×10^6 cells in 10 cm plates were transfected with DNA (4 µg/plate) using Lipofectamine Plus reagent. At 48 h post-transfection, cells were harvested and the total RNAs were extracted using an RNeasy Mini Kit. Potential contaminating plasmid DNA in the total RNAs was digested with RNase-free DNase.

Ribonuclease Protection Assay (RPA) was performed using the RPA III kit according to the manufacturer's instruction. To prepare RNA probe, the region between –277 and –1 of human p27 5'-UTR sequence and +1 to +133 in the ORF of the firefly luciferase gene was amplified using PCR and cloned into pGEM-T Easy vector. The resulting plasmid was linearized with SpeI and labeled *in vitro* using T7 RNA polymerase in the presence of 0.5 mM each of ATP, UTP, GTP and 0.01 mM CTP supplemented with 50 µCi [α -³²P]CTP. The [α -³²P]labeled probe was digested with DNase I and purified using a Sephadex G-25 Quick Spin Column. About 5×10^5 c.p.m. of probe was hybridized to 15 µg total RNA at 45°C overnight followed by digestion with RNase T1/A for 35 min at 37°C. The reaction was then stopped and the protected RNA samples were separated by electrophoresis on a 6% acrylamide/8 M urea gel. The gel was then dried for autoradiography.

RESULTS

The 5'-UTR sequence of p27 enhances the expression of the second cistron in the dicistronic DNA test

Dicistronic DNA test has been used in the previous studies to show the existence of IRES activities in the 5'-UTR sequence of both human and murine p27, although it was thought that the length of the IRES element may vary between the two species (8,9). To further characterize the 5'-UTR sequence, we engineered three dicistronic constructs containing various lengths of the 5'-UTR sequence of human p27 (Figure 1B) flanked by *Renilla* and firefly luciferase genes (Figure 1A). These constructs were then transfected into H1299 cells for the analysis of potential IRES activity using luciferase assay. As shown in Figure 1C and Table 1, the expression of the second cistron firefly luciferase was greatly enhanced by the presence of the p27 5'-UTRs although the enhancement decreased gradually with the truncation of sequence from the 5' end. The expression of *Renilla* luciferase was not changed by the insertion of the 5'-UTR sequence of p27. The increased ratio of firefly to *Renilla* luciferase activities by the 5'-UTR sequence of p27 compared with vector control suggests that there may be an IRES element in these sequences (Figure 1D). Insertion of a hairpin in front of the first cistron did not affect the expression of the second cistron firefly luciferase (data not shown). These observations are consistent with the previous findings and with the claim of the existence of IRES activity in the 5'-UTR sequence of p27 (8–10).

The 5'-UTR of p27 does not display an IRES activity in the dicistronic mRNA assay

The above studies suggest that the 5'-UTR of p27 may contain an IRES element. However, the enhanced expression of the second cistron could also be due to the existence of promoters in the 5'-UTR or due to alternative splicing of the dicistronic transcripts as suggested previously (15–17,19). To further determine whether the 5'-UTR of p27 contains IRES activities, we performed a more stringent dicistronic mRNA test, which allows a direct analysis whether the 5'-UTR sequence of p27 in the intergenic region can increase the translation of the second cistron firefly luciferase without the transcriptional and splicing interference. For this purpose, we engineered additional constructs for making *in vitro* dicistronic transcripts containing 5'-caps and 3'-poly(A) tails (Figure 2A). In addition, a well-known viral HRV IRES sequence was also cloned into the intergenic region to serve as a positive control. These *in vitro* transcripts were then transfected into H1299 cells, which were then harvested for luciferase assay. As shown in Figure 2B and C, the expression of the second cistron firefly luciferase was enhanced only by the HRV IRES as expected. None of the 5'-UTR sequences of p27 enhanced the expression of the second cistron firefly luciferase. Thus, it is likely that the 5'-UTR of p27 does not contain a functional IRES element as previously thought.

The 5'-UTR sequence of p27 contains functional promoter elements in promoterless dicistronic DNA assay

The above results of dicistronic mRNA assay prompted us to investigate whether the 5'-UTR sequence of p27 contains

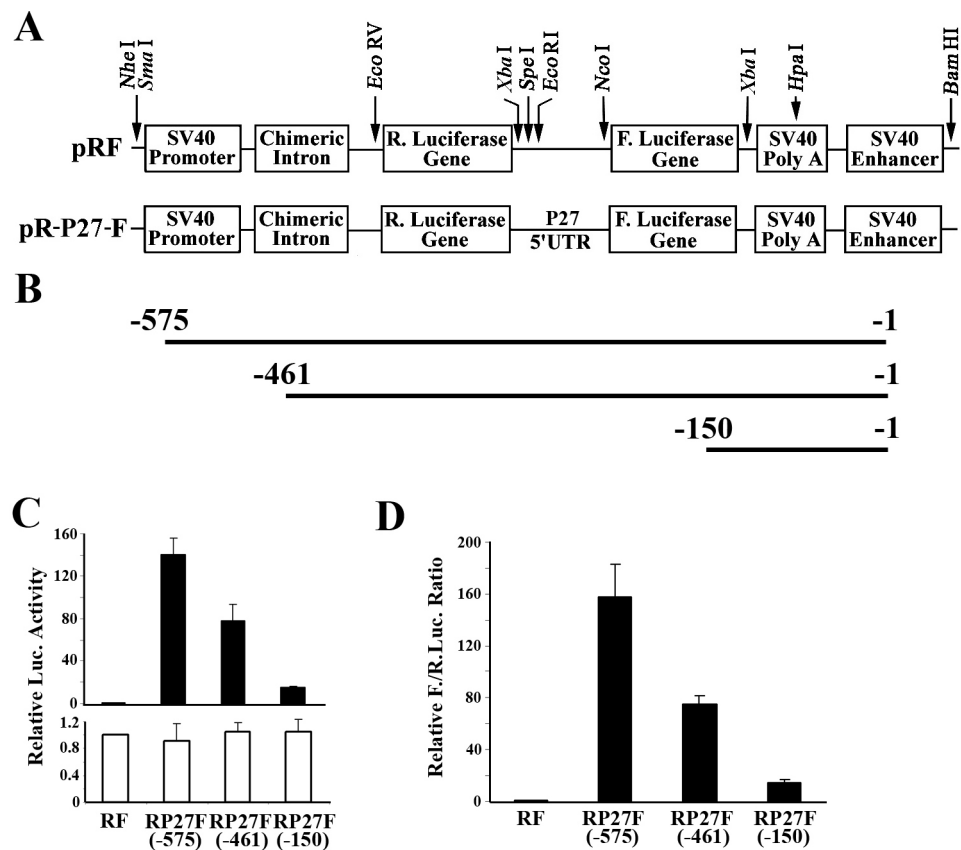


Figure 1. Dicistronic DNA test of the 5'-UTR sequence of human p27. (A) Schematic diagram of dicistronic DNA constructs without (pRF) or with the insert of 5'-UTR of p27 (pR-P27-F) in the intergenic region. The locations of several relevant restriction enzyme sites are shown by arrows. (B) Schematic diagram of deletion of the 5'-UTR sequence of p27. The deletions were from the 5' end of the sequence with the positions of deletion indicated. (C) Relative luciferase activities generated by the dicistronic DNA constructs. H1299 cells were transfected with pRF and pR-P27-F constructs. Twenty-four hours following transfection, cells were harvested and the *Renilla* and firefly luciferases activities were measured. Firefly (filled bars) and *Renilla* (open bars) luciferase activities were normalized to the co-transfected β -galactosidase activity and then to that generated by the vector (RF). (D) Relative F/R luciferase activity ratios. The relative ratios of firefly and *Renilla* luciferase activities were calculated and normalized to that of the vector-transfected cells (RF). The data were from one of the four independent experiments. R, *Renilla*; F, firefly.

Table 1. Luciferase activities of various constructs in H1299 and 293T cells

	RF	RP27F(−575)	RP27F(−461)	RP27F(−150)
Fluc (+P) ^a	485 ^b	75 576	46 340	7836
Rluc (+P) ^a	132 334	164 179	157 607	167 983
Fluc (−P) ^a	293	82 575	34 486	6893
Rluc (−P) ^a	372	333	274	407
Fluc (−P) ^c	519	611 571	98 286	3858
Rluc (−P) ^c	435	417	344	413

^aLuciferase activities were measured from lysates prepared from H1299 cells transfected with various constructs and the values were normalized with β -galactosidase activities to adjust transfection efficiency. +P and −P represent constructs with and without the vector promoter, respectively.

^bThe data presented in this table are from one representative experiment.

^cLuciferase activities were measured from lysates prepared from 293T cells transfected with various constructs and the values were normalized with β -galactosidase activities to adjust transfection efficiency.

a promoter that could be responsible for the observed stimulation of expression of the second cistron firefly luciferase in the dicistronic DNA assays (Figure 1). For this purpose, we first engineered promoterless dicistronic constructs as

described previously (19,20) by simply removing the unique SV40 promoter together with the intron sequence from the pRF-based dicistronic constructs (Figure 3A). These promoterless dicistronic constructs will not generate dicistronic mRNAs after transfection into cells due to the lack of the SV40 promoter. Thus, the activity of any potential IRES element in the constructs should not be detected. Any expression of the second cistron firefly luciferase from these promoterless dicistronic DNAs will be due to the existence of a promoter in the 5'-UTR sequence of p27. As shown in Figure 3B and C, the enhanced expression profile of the second cistron firefly luciferase in the promoterless construct is similar to that generated in the presence of the SV40 promoter (compare with Figure 1). The actual firefly luciferase activities are similar to that generated in the presence of SV40 promoter, whereas the actual *Renilla* luciferase activity is drastically decreased due to the removal of the vector promoter (see Table 1). Thus, the 5'-UTR sequence of p27 likely contains promoters.

To ensure that the above observations are not specific to the cell line used, we performed similar studies using 293T cells, which has been used in a previous study of p27 5'-UTR (25). First, dicistronic mRNAs were transfected into 293T cells and

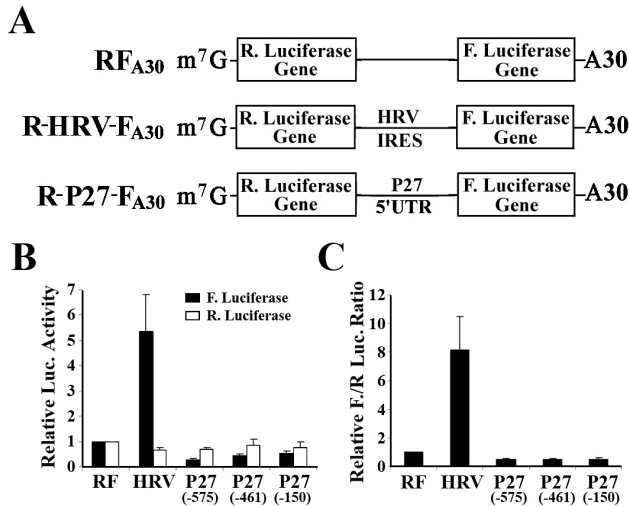


Figure 2. Dicistronic mRNA test of the 5'-UTR of human p27 in H1299 cells. (A) Schematic diagram of the dicistronic mRNA used for translation in H1299 cells. *In vitro* transcripts with 5'-cap (m⁷GpppG) and 3'-poly(A) tail (A₃₀) were synthesized using Sp6 RNA polymerase from linearized vector alone (RF_{A30}), constructs containing the IRES of HRV (R-HRV-F_{A30}) or the 5'-UTRs of p27 (R-P27-F_{A30}). (B) Relative luciferase activities from dicistronic mRNAs in H1299 cells. H1299 cells were transfected with the dicistronic mRNAs, and 8 h following transfection *Renilla* and firefly luciferase activities were measured and normalized to that of vector control (RF). (C) Relative F/R luciferase activity ratios. The ratios of relative firefly and *Renilla* luciferase activities were calculated and normalized to that of the control vector-transfected cells (RF).

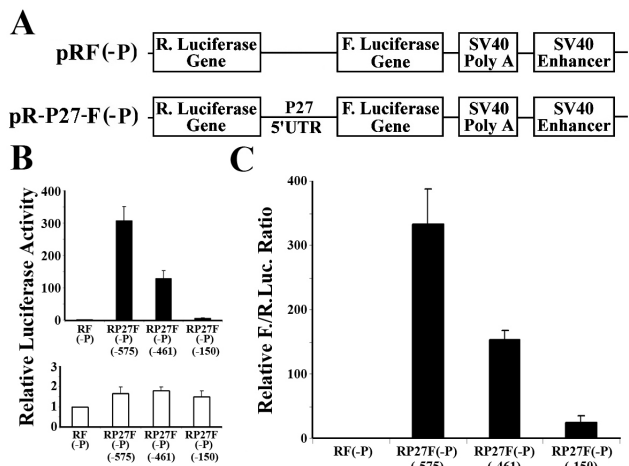


Figure 3. Promoterless dicistronic DNA test of the 5'-UTR sequence of human p27. (A) Schematic diagram of promoterless dicistronic DNA constructs without insert [pRF(-P)] or with the 5'-UTR of p27 [pR-P27-F(-P)] in the intergenic region. (B) Relative luciferase activity generated by the promoterless dicistronic DNA constructs. H1299 cells were transfected with pRF(-P) and pR-P27-F(-P) constructs. Twenty-four hours following transfection, cells were harvested and the *Renilla* and firefly luciferases activities were measured. The firefly (filled bars) and *Renilla* (open bars) luciferase activities were normalized to the co-transfected β -galactosidase activity and then to that generated by the vector (RF). (C) The relative ratios were calculated and normalized to that of the vector-transfected cells [RF(-P)]. The data were from one of the four independent experiments. R, *Renilla*; F, firefly.

luciferase activities were determined as described above. As shown in Figure 4A and B, the expression of the second cistron firefly luciferase was enhanced only by the HRV IRES as expected. None of the 5'-UTR sequences of p27 enhanced

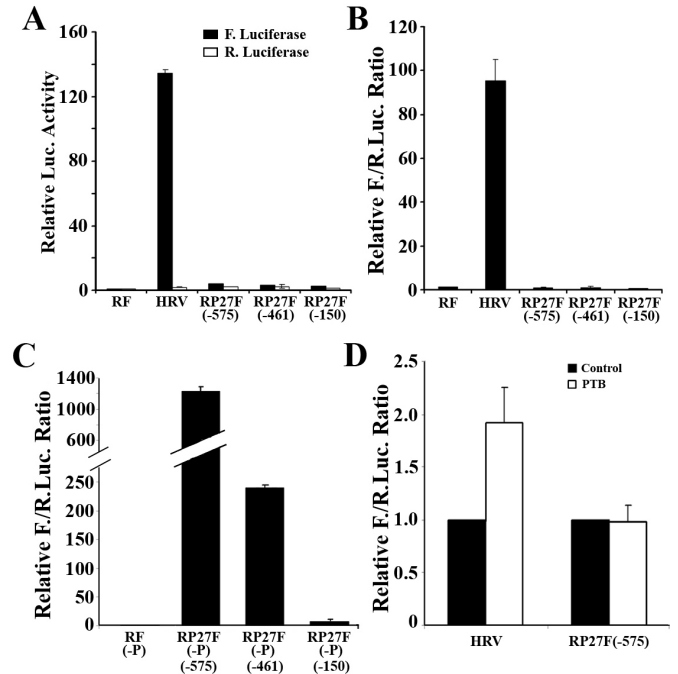


Figure 4. Dicistronic mRNA and promoterless dicistronic DNA test in 293T cells and *in vitro*. (A and B) Relative luciferase activity from dicistronic mRNAs in 293T cells. *In vitro* dicistronic transcripts were prepared as described in Figure 2 and transfected into 293T cells. Eight hours following transfection, *Renilla* and firefly luciferase activities were measured and normalized to the co-transfected β -galactosidase and then to the vector control (RF) (A). The ratios of relative firefly and *Renilla* luciferase activities were calculated and normalized to that of the control vector-transfected cells (RF) (B). (C) Relative luciferase activities from promoterless dicistronic DNAs in 293T cells. 293T cells were transfected with promoterless dicistronic constructs and luciferase activities were determined as described in Figure 3. Firefly and *Renilla* luciferase activities were normalized to the co-transfected β -galactosidase activity and then to the vector control [RF(-P)]. The relative ratios were calculated and normalized to that of the vector-transfected cells [RF(-P)]. (D) Relative luciferase activity from dicistronic mRNAs *in vitro*. Dicistronic mRNAs containing the full-length 5'-UTR (575 bases) of p27 or the HRV IRES in the intergenic region were used to program translation in RRL in the absence or presence of recombinant PTB as described in Materials and Methods. Firefly and *Renilla* luciferase activities were determined and the relative ratios were calculated and normalized to the control in the absence of PTB.

the expression of the second cistron firefly luciferase in 293T cells. These observations are consistent with the data shown using H1299 cells (compare with Figure 2). We next transfected the promoterless dicistronic constructs into 293T cells and determined luciferase activities. As shown in Figure 4C, the firefly luciferase expression was enhanced by the presence of the 5'-UTR sequence of p27 in the intergenic region, suggesting the presence of promoter activities (see also Table 1). This observation is also similar to that generated in H1299 cells (compare with Figure 3). In fact, the promoter activity in the longest form of 5'-UTR of p27 as indicated by the firefly luciferase activity is much more pronounced in 293T cells compared with H1299 cells, although the shorter 5'-UTR segments had similar activities in the two cell lines (see Table 1). Similar observations have also been made with HeLa cells that were used in two other previous studies (9,10). Taken together, we conclude that the previous findings of the putative 'IRES' activities in the 5'-UTR sequence of p27 was not due to the

Table 2. Luciferase activities of dicistronic RNAs generated in RRL

	HRV (–PTB)	HRV (+PTB)	p27(–PTB)	p27(+PTB)
Fluc	1898 ^a	4626	2123	2179
Rluc	988 828	1 380 389	1 057 596	1 207 663

^aThe data presented in this table are from one representative experiment.

specific cell lines used and that most likely they are all misled from the promoter activities.

Recently, it was suggested that PTB enhanced the putative IRES activity of p27 5'-UTR in cell-free expression system and *in vivo* (25). We performed similar studies by translating dicistronic RNA transcripts in RRL in the absence or presence of purified PTB with HRV IRES as a positive control. Previously, we and others have observed that the HRV IRES is inactive in RRL unless HeLa cell extracts or PTB is added (19,26,27) (Figure 4D and Table 2). Contrary to the previous finding by Cho *et al.* (25), we did not observe any enhanced expression of the second cistron firefly luciferase by the 5'-UTR of p27 in the absence or presence of PTB (Figure 4D). In fact, the firefly luciferase activity generated is very low, >500-fold less than the *Renilla* luciferase activity (Table 2).

Existence of promoters in the 5'-UTR of p27 as revealed by RNase protection assay

To confirm the above conclusion that the 5'-UTR of p27 contains promoters, we further tested if the transcripts with only the second cistron firefly luciferase exist in the cells transfected with dicistronic DNA constructs. RNase protection assay was performed on total RNAs isolated from H1299 cells following transfection with the traditional and promoterless dicistronic constructs using a RNA probe covering the region between –277 and –1 of human p27 5'-UTR sequence and the region between +1 and +133 of firefly luciferase ORF (Figure 5A). As shown in Figure 5B, a short fragment with an estimated size of 124 bases was produced from the traditional dicistronic vector (lane 4), which represents the protected luciferase gene from the dicistronic RNA transcripts. This product is barely detectable with the RNA isolated from cells transfected with the promoterless dicistronic vector (lane 8), suggesting that the functional SV40 promoter has been successfully removed. A fragment with an estimated size of 415 bases was produced from the traditional dicistronic constructs containing the 575 and 461 bases of p27 5'-UTRs (lanes 5 and 6). This product is likely generated from the dicistronic RNA produced using the vector SV40 promoter. However, a similar product, albeit with less intensity, was also observed with the promoterless dicistronic construct (lane 9), suggesting that a transcription start site may exist upstream of the probe used (Figure 5A, the marker X). Four fragments of 272, 258, 184 and 166 bases were also produced with both the traditional and the promoterless dicistronic constructs containing 575 and 461 bases of p27 5'-UTRs (lanes 5, 6, 9 and 10), suggesting that four transcription start sites likely exist at around –139, –125, –51 and –33. Interestingly, for the traditional dicistronic construct containing only 150 bases of the p27 5'-UTR (lane 7), only the product of 184 bases was observed, suggesting that except the transcription start

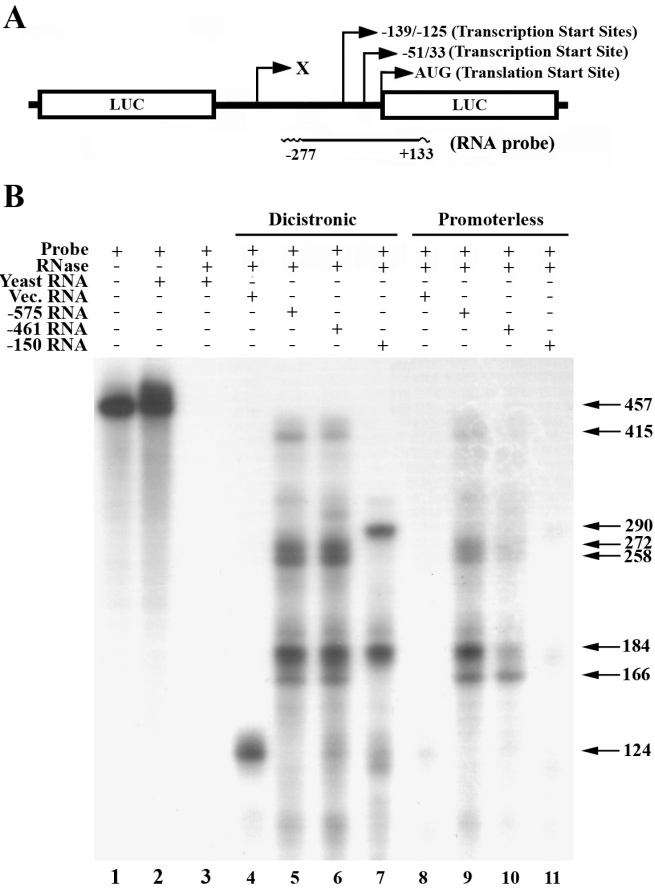


Figure 5. RNase protection assay of p27 5'-UTR in dicistronic constructs. (A) Schematic diagram illustrating the location of transcription start sites and the probe used for RNase protection assay. X indicates an upstream transcription start site. (B) RNase protection assay. Total RNAs were isolated from H1299 cells following transfection with dicistronic vector pRF (lane 4), pR-P27-F (lanes 5–7), promoterless dicistronic vector pRF(–P) (lane 8), and promoterless pR-P27-F (lanes 9–11) and then used for RNase protection assay as described in Materials and Methods. Lanes 1–3 are control reactions without (lanes 1 and 2) or with (lane 3) RNase in the absence (lane 1) or presence (lanes 2–3) of yeast RNA.

site –51, all others are silent in this construct. The product of 290 bases (lane 7) was likely generated from the protection of the dicistronic RNA transcripts containing 150 bases of p27 5'-UTR. It appears that the shortest 5'-UTR segment (–150) in the promoterless construct apparently has promoter activity (Figure 3) but it is too weak for direct RNA analysis to detect the transcript (Figure 5, lane 11). It is possible that the transcription start site at –51 requires the vector SV40 promoter and/or the upstream p27 promoters, which may function as regulatory promoters (28). It is also possible that the production of the 184 bases fragment from the shortest traditional dicistronic construct was due to potential alternative splicing, which is absent with the removal of sequences together with the SV40 promoter in the shortest promoterless dicistronic plasmid (see below).

To further confirm the promoter activities in the 5'-UTR sequence of p27, we cloned these sequences into the standard promoter-testing monocistronic vector pGL3, which does not have any promoter element. These constructs containing the different sizes of p27 5'-UTR were then transfected into

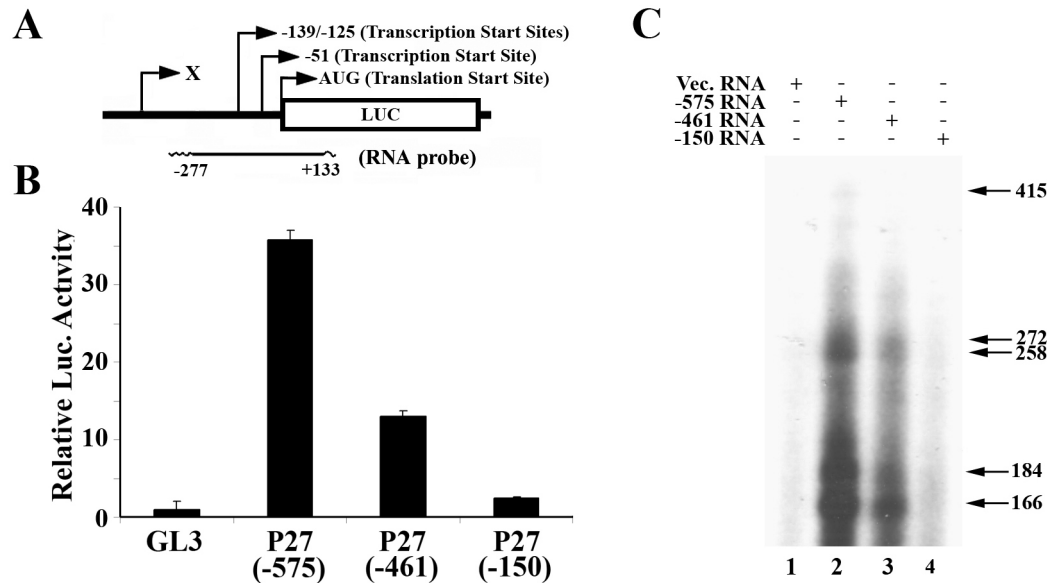


Figure 6. Promoter activity of p27 5'-UTR in monocistronic constructs and RNase protection assay. (A) Schematic diagram illustrating the location of transcription start sites and the probe used for RNase protection assay. X indicates an upstream transcription start site. (B) Luciferase assay. H1299 cells were transfected with vector pGL3 and monocistronic constructs that harboring the 5'-UTR sequences of p27. Forty-eight hours following transfection, cells were harvested and luciferase activities were measured. The luciferase activities were then normalized to the co-transfected β -galactosidase activity and then to that generated by the vector (pGL3). (C) RNase protection assay. Total RNAs were isolated following transfection with the vector pGL3 (lane 1) and constructs containing the 5'-UTR sequences of p27 (lanes 2–4) and then used for RNase protection assay as described in Materials and Methods.

H1299 cells for the determination of luciferase activity. As shown in Figure 6B, similar firefly luciferase activities were observed with these constructs compared with that generated from the traditional and promoterless dicistronic constructs (compare with Figures 1 and 3). RNase protection analysis of the RNAs isolated from cells transfected with these monocistronic constructs also showed the production of various transcription products similar to that shown with the dicistronic constructs (compare Figure 6C with Figure 5) although, again, the shortest 5'-UTR segment had promoter activity which is too weak to be detected by direct RNA analysis.

Based on the above observations with promoterless dicistronic and the standard promoter-testing monocistronic constructs, we conclude that the 5'-UTR sequence of human p27 contains promoter activities that can initiate transcription at several sites. Transcription at these sites in the dicistronic DNA assay generates monocistronic transcripts containing only the firefly luciferase gene with short 5'-UTRs that can be translated easily using the scanning mechanism (12,13). These shorter monocistronic RNA transcripts are likely responsible for the enhanced expression of firefly luciferase in the dicistronic DNA test and they may represent the previously reported IRES activity.

DISCUSSION

In this work, we studied the 5'-UTR sequence of human p27 mRNA and further tested the putative IRES activity using promoterless dicistronic and monocistronic vectors and direct assay of dicistronic mRNAs. Our results indicated that the 5'-UTR sequence of p27 contains promoters that can drive production of abundant monocistronic transcripts with shorter

5'-UTRs. These findings raise questions on the IRES activity of the 5'-UTR sequence of p27 reported previously using the traditional dicistronic DNA assay.

Use of IRES for translating the untranslatable cellular mRNAs with long 5'-UTRs is an interesting concept (29). The use of such a mechanism for translating polycistronic viral RNAs has been convincingly demonstrated previously (30). However, whether cellular mRNAs also use this mechanism is currently under debate (15,31). The central problem associated with the majority of studies claiming cellular IRES is the use of traditional dicistronic DNA test which does not directly test the translation. Potential promoter activities and alternative splicing could both contribute to the observed 'IRES' activity. These potential problems have recently been demonstrated on several previously believed-to-be cellular IRES elements. These IRES activities were shown to be due to cryptic promoters present in the 5'-UTRs (19,23,32–34) or due to differential splicing (35) which would create smaller monocistronic transcripts from the dicistronic DNAs for potential cap-dependent translation initiation of the second cistron. In one of these studies, the prevailing cellular IRES sequence of eIF4G (36,37) was shown to be an C/E BP beta transcription factor binding site (19). In another study, the strong IRES activity in the 5'-UTR of XIAP mRNAs was found to be mostly due to alternative splicing (35). Furthermore, a promoter activity was also found in the 5'-UTR sequence of hepatitis C virus, which has previously been shown to have IRES activities (38). While these studies do not necessarily exclude in general the existence of IRES in cellular mRNAs *per se*, they necessitate more stringent studies such as tests using promoterless dicistronic DNA transfection, dicistronic RNA transfection and RT-PCR for safeguarding any claims of cellular IRES.

Previously, the 5'-UTR sequences of both human and murine p27 were reported to contain IRES activities using traditional dicistronic DNA assays (8,9). While the IRES in the mouse p27 was thought to be located in the 217 bases proximal to the AUG start codon, the full IRES activity in human p27 was thought to be located within the 356 bases upstream of the start codon. However, in the present study, we showed that these previously reported IRES activities are likely due to the promoters present in the 5'-UTR sequence of p27 (see below). In the case of human p27, the deletion analysis showed that the 184 bases upstream of the AUG start codon contains about half of the putative IRES activity with a 15-fold stimulation of Fluc/Rluc ratio by the 5'-UTR of p27 compared with the vector (9). This extent of stimulation is similar to the promoter activity we found using the dicistronic construct containing the 150 bases of human p27 5'-UTR (see below). Furthermore, no stimulation of the second cistron was observed with the 5'-UTR of p27 in the dicistronic RNA assay (Figure 2). In fact, the translation of the second cistron was inhibited in the dicistronic RNA assay by inserting the 5'-UTR sequence of p27 into the intergenic region. These observations further suggest that the 5'-UTR of human p27 may not contain any significant IRES element as previously thought.

Using both the promoterless dicistronic vector and the standard promoter-testing monocistronic vector pGL3, we demonstrated that the 5'-UTR of p27 contains promoters to stimulate the transcription of its following sequences. Because deletion of the first 114 bases reduced by about half the activity to stimulate the expression of firefly luciferase gene, it is likely that the 575 bases of the 5'-UTR sequence of p27 contain a promoter at the 5' end, which is absent in the construct harboring the 3' 461 bases of the 5'-UTR sequence. Indeed, we found by RNase protection assay that a transcription start site exists upstream of our probe (marked as X in Figure 5A). Furthermore, Kullmann *et al.* (9) also found that the 575 bases of the human p27 5'-UTR contains a promoter that is absent in the 461 bases of p27 5'-UTR, consistent with our findings. Further deletion at the 5' end from -461 to -150 drastically reduced the activity in stimulating the luciferase expression. This observation suggests that the cryptic promoters likely exist in the region between -461 and -150. This conclusion is confirmed by RNase protection assay, which showed that two transcription start sites at positions -125 and -139 are present in both the 575 and 461 constructs but they are missing in the -150 construct (Figure 5). It is, however, noteworthy that the remaining 150 bases in the 5'-UTR of human p27 still stimulate the expression of the luciferase reporter albeit at a much reduced level compared with the longer sequences, suggesting that another promoter likely exists in the 150 bases upstream of the AUG start codon. RNase protection assay confirmed that there is indeed a transcription start site at about -51 in all three constructs. However, this promoter activity appears to be very weak in the absence of upstream promoters, such as the vector SV40 promoter or promoters present in the upstream sequence of p27 5'-UTR. It, thus, remains to be determined whether these putative promoters have any physiological roles in regulating p27 expression. The use of these promoters may generate transcripts with shorter 5'-UTRs, which can be efficiently translated using the cap-dependent scanning model for translation initiation. Production of these shorter transcripts may be

important for the increased expression of p27 during G1 phase. We are currently testing these possibilities and working toward this direction.

Protein factors have been suggested to be involved in the putative 'IRES' activity of p27. Several mRNA-binding proteins, HuR, HuD and hnRNP C1/C2 (7,9), and more recently PTB (25), were found to interact with the 5'-UTR of p27 and shown to either repress or enhance p27 expression. However, using *in vitro* translation system we found no evidence that PTB stimulates the expression of the second cistron firefly luciferase of the dicistronic RNA containing the 5'-UTR of p27. Furthermore, the finding of cryptic promoters in the 5'-UTR sequence of p27 urges the need to re-evaluate the mechanism how these factors regulate p27 expression. Because all the previous claims are based on transfections using DNA reporter constructs, the effect of these factors on transcription using the promoters in the 5'-UTR of p27 could not be ruled out. Thus, the effect of these factors on the expression of endogenous p27 might in fact be mediated through mechanisms other than the putative 'IRES'. HuR and HuD are the AU-rich element-binding protein (39), which may affect the p27 expression through binding to the 3'-UTR of p27 mRNA. However, PTB and hnRNP-C (40,41) are the known factors associated with RNA splicing and they may affect the expression of p27 by affecting the splicing of p27 mRNAs. Sequence examination showed that the 5'-UTR of p27 contains two potential splicing acceptor sites close to the AUG start codon. One is located at -114 to -129, while the other is at -37 to -52. Alternatively, these factors may affect the expression of other unknown factors, which indirectly contributes to p27 expression regulation. We propose that the effect of these factors on p27 expression need to be re-evaluated in the future by performing more stringent experiments, such as using transfection of RNA transcripts instead of DNA constructs and by testing the possibility of their effects on transcription and splicing of p27 mRNAs.

ACKNOWLEDGEMENTS

The authors thank James G. Patton at Vanderbilt University for the pET28aPTB plasmid. This work was supported in part by the National Institutes of Health grant CA94961 and by the Department of Defense grant DAMD17-02-1-0073. Funding to pay the Open Access publication charges for this article was provided by National Institutes of Health.

Conflict of interest statement. None declared.

REFERENCES

- Hengst, L. and Reed, S.I. (1998) Inhibitors of the Cip/Kip family. *Curr. Top. Microbiol. Immunol.*, **227**, 25–41.
- Sherr, C.J. and Roberts, J.M. (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.*, **13**, 1501–1512.
- Millard, S.S., Yan, J.S., Nguyen, H., Pagano, M., Kiyokawa, H. and Koff, A. (1997) Enhanced ribosomal association of p27(Kip1) mRNA is a mechanism contributing to accumulation during growth arrest. *J. Biol. Chem.*, **272**, 7093–7098.
- Agrawal, D., Hauser, P., McPherson, F., Dong, F., Garcia, A. and Pledger, W.J. (1996) Repression of p27kip1 synthesis by platelet-derived growth factor in BALB/c 3T3 cells. *Mol. Cell. Biol.*, **16**, 4327–4336.

5. Hengst, L. and Reed, S.I. (1996) Translational control of p27Kip1 accumulation during the cell cycle. *Science*, **271**, 1861–1864.
6. Keene, J.D. (1999) Why is Hu where? Shuttling of early-response-gene messenger RNA subsets. *Proc. Natl Acad. Sci. USA*, **96**, 5–7.
7. Millard, S.S., Vidal, A., Markus, M. and Koff, A. (2000) A U-rich element in the 5' untranslated region is necessary for the translation of p27 mRNA. *Mol. Cell. Biol.*, **20**, 5947–5959.
8. Miskimins, W.K., Wang, G., Hawkinson, M. and Miskimins, R. (2001) Control of cyclin-dependent kinase inhibitor p27 expression by cap-independent translation. *Mol. Cell. Biol.*, **21**, 4960–4967.
9. Kullmann, M., Gopfert, U., Siewe, B. and Hengst, L. (2002) ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5'UTR. *Genes Dev.*, **16**, 3087–3099.
10. Gopfert, U., Kullmann, M. and Hengst, L. (2003) Cell cycle-dependent translation of p27 involves a responsive element in its 5'-UTR that overlaps with a uORF. *Hum. Mol. Genet.*, **12**, 1767–1779.
11. Sonenberg, N. (1994) Regulation of translation and cell growth by eIF-4E. *Biochimie*, **76**, 839–846.
12. Kozak, M. (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.*, **266**, 19867–19870.
13. Kozak, M. and Shatkin, A.J. (1978) Identification of features in 5' terminal fragments from reovirus mRNA which are important for ribosome binding. *Cell*, **13**, 201–212.
14. Merrick, W.C. (2004) Cap-dependent and cap-independent translation in eukaryotic systems. *Gene*, **332**, 1–11.
15. Kozak, M. (2001) New ways of initiating translation in eukaryotes? *Mol. Cell. Biol.*, **21**, 1899–1907.
16. Kozak, M. (2003) Alternative ways to think about mRNA sequences and proteins that appear to promote internal initiation of translation. *Gene*, **318**, 1–23.
17. Kozak, M. (2002) Pushing the limits of the scanning mechanism for initiation of translation. *Gene*, **299**, 1–34.
18. Stoneley, M., Subkhankulova, T., Le Quesne, J.P., Coldwell, M.J., Jopling, C.L., Belsham, G.J. and Willis, A.E. (2000) Analysis of the c-myc IRES; a potential role for cell-type specific trans-acting factors and the nuclear compartment. *Nucleic Acids Res.*, **28**, 687–694.
19. Han, B. and Zhang, J.T. (2002) Regulation of gene expression by internal ribosome entry sites or cryptic promoters: the eIF4G story. *Mol. Cell. Biol.*, **22**, 7372–7384.
20. Han, B., Dong, Z., Liu, Y., Chen, Q., Hashimoto, K. and Zhang, J.T. (2003) Regulation of constitutive expression of mouse PTEN by the 5'-untranslated region. *Oncogene*, **22**, 5325–5337.
21. Zhang, J.T. and Ling, V. (1991) Study of membrane orientation and glycosylated extracellular loops of mouse P-glycoprotein by *in vitro* translation. *J. Biol. Chem.*, **266**, 18224–18232.
22. Perez, I., McAfee, J.G. and Patton, J.G. (1997) Multiple RRM's contribute to RNA binding specificity and affinity for polypyrimidine tract binding protein. *Biochemistry*, **36**, 11881–11890.
23. Han, B., Dong, Z. and Zhang, J.T. (2003) Tight control of platelet-derived growth factor B/c-sis expression by interplay between the 5'-untranslated region sequence and the major upstream promoter. *J. Biol. Chem.*, **278**, 46983–46993.
24. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
25. Cho, S., Kim, J.H., Back, S.H. and Jang, S.K. (2005) Polypyrimidine tract-binding protein enhances the internal ribosomal entry site-dependent translation of p27Kip1 mRNA and modulates transition from G1 to S phase. *Mol. Cell. Biol.*, **25**, 1283–1297.
26. Hunt, S.L. and Jackson, R.J. (1999) Polypyrimidine-tract binding protein (PTB) is necessary, but not sufficient, for efficient internal initiation of translation of human rhinovirus-2 RNA. *RNA*, **5**, 344–359.
27. Borman, A., Howell, M.T., Patton, J.G. and Jackson, R.J. (1993) The involvement of a spliceosome component in internal initiation of human rhinovirus RNA translation. *J. Gen. Virol.*, **74**, 1775–1788.
28. Carey, M. (1998) The enhanceosome and transcriptional synergy. *Cell*, **92**, 5–8.
29. Vagner, S., Galy, B. and Pyronnet, S. (2001) Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. *EMBO Rep.*, **2**, 893–898.
30. Pe'ery, T. and Mathews, M.B. (2000) Chapter 8. In Sonenberg, N., Hershey, J.W. and Mathews, M.B. (eds), *Translational control of gene expression*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 371–424.
31. Schneider, R., Agol, V.I., Andino, R., Bayard, F., Cavener, D.R., Chappell, S.A., Chen, J.J., Darlix, J.L., Dasgupta, A., Donze, O. *et al.* (2001) New ways of initiating translation in eukaryotes? *Mol. Cell. Biol.*, **21**, 8238–8246.
32. Hecht, K., Bailey, J.E. and Minas, W. (2002) Polycistronic gene expression in yeast versus cryptic promoter elements. *FEMS Yeast Res.*, **2**, 215–224.
33. Verge, V., Vonlanthen, M., Masson, J.M., Trachsel, H. and Altmann, M. (2004) Localization of a promoter in the putative internal ribosome entry site of the *Saccharomyces cerevisiae* TIF4631 gene. *RNA*, **10**, 277–286.
34. Wang, Z., Weaver, M. and Magnuson, N.S. (2005) Cryptic promoter activity in the DNA sequence corresponding to the pim-1 5'-UTR. *Nucleic Acids Res.*, **33**, 2248–2258.
35. Van Eden, M.E., Byrd, M.P., Sherrill, K.W. and Lloyd, R.E. (2004) Demonstrating internal ribosome entry sites in eukaryotic mRNAs using stringent RNA test procedures. *RNA*, **10**, 720–730.
36. Gan, W., Celle, M.L. and Rhoads, R.E. (1998) Functional characterization of the internal ribosome entry site of eIF4G mRNA. *J. Biol. Chem.*, **273**, 5006–5012.
37. Gan, W. and Rhoads, R.E. (1996) Internal initiation of translation directed by the 5'-untranslated region of the mRNA for eIF4G, a factor involved in the picornavirus-induced switch from cap-dependent to internal initiation. *J. Biol. Chem.*, **271**, 623–626.
38. Dumas, E., Staedel, C., Colombat, M., Reigadas, S., Chabas, S., Astier-Gin, T., Cahour, A., Litvak, S. and Ventura, M. (2003) A promoter activity is present in the DNA sequence corresponding to the hepatitis C virus 5'-UTR. *Nucleic Acids Res.*, **31**, 1275–1281.
39. Brennan, C.M. and Steitz, J.A. (2001) HuR and mRNA stability. *Cell Mol. Life Sci.*, **58**, 266–277.
40. Choi, Y.D., Grabowski, P.J., Sharp, P.A. and Dreyfuss, G. (1986) Heterogeneous nuclear ribonucleoproteins: role in RNA splicing. *Science*, **231**, 1534–1539.
41. Patton, J.G., Mayer, S.A., Tempst, P. and Nadal-Ginard, B. (1991) Characterization and molecular cloning of polypyrimidine tract-binding protein: a component of a complex necessary for pre-mRNA splicing. *Genes Dev.*, **5**, 1237–1251.